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Urine test for the assessment of smoking status

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(Accepted 15 January 2001)

Abstract: A simple, quick and inexpensive test for smoking status would be useful in a variety of settings. The non-polar barbituric acid derivative 1,3-dibutyl-2-thiobarbituric acid (DBTB) is described as a novel derivatisation reagent for nicotine and its metabolites in the König reaction to assess smoking status. The relative performance of qualitative methods for assessing smoking status using DBTB and the previously employed derivatisation reagent 1,3-diethyl-2-thiobarbituric acid (DETB), as well as quantitatively-based methods for determining 'total nicotine metabolites' (TNMs) using these two reagents, were evaluated against a cotinine-based radioimmunoassay (RIA) as the 'gold standard'. Clinical sensitivity and specificity for all the approaches studied were in excess of 94%. Simple qualitative assessment by eye was superior to quantitatively-based measures of smoking status. Correlation between estimation of nicotine metabolites using DBTB, DETB and RIA were good. The most efficient and convenient method to distinguish between smokers and non-smokers was the simple qualitative method using the more lipophilic reagent DBTB.

Key words: Cotinine. König reaction. Smoking. Total nicotine metabolites.

Introduction

Smoking is the major preventable cause of mortality and morbidity in the western world. Recent evidence suggests that about half of all regular smokers will die from their smoking habit.¹ Whilst smoking prevalence is on the decline within the industrialised nations, it is increasing in most other parts of the world. It has been suggested² that the most effective strategy for reducing the incidence of cardiovascular disease, stroke and cancer is to cease smoking.

The efficiency with which smokers smoke their cigarettes varies between individuals, and smokers often deny their addiction. Smoking cessation initiatives, the monitoring of smoking during pregnancy,³ and epidemiological studies of smoking-related illness would be facilitated if simple, reliable methods were available to identifying smokers and assess their daily intake of tobacco-related compounds.

For many years, questionnaires have provided information on individual smoking habits; however,

often they have been found to be incorrect when compared with measurements taken in a laboratory. The most widely used marker of tobacco usage has been the measurement of cotinine, the primary nicotine metabolite. This biomarker has been used largely because of its long serum half-life (about 17 hours), compared with a half-life of about two hours for nicotine.⁴ Methods for cotinine measurement include gas chromatography-mass spectrometry (GC-MS),^{5,6} high-performance liquid chromatography (HPLC)⁷⁻⁹ and immunoassay;^{10,11} these have been applied to a wide variety of biological samples including serum, saliva, urine and hair.

GC-MS is the most sensitive and specific method for the measurement of individual nicotine metabolites, but it is labour-intensive and requires very expensive equipment. HPLC lacks sensitivity and generally picks out only a few of the individual metabolites. Immunoassays can be extremely sensitive but measure only cotinine with variable cross-reactivity to other metabolites, especially the 3'- and 5'-hydroxycotinine metabolites, depending on the antibody employed.

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RWLP0278

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Alternative urine-based methods to identify smokers and assess their relative nicotine intakes involve the use of modifications (or variants) of the simple and inexpensive colorimetric König reaction, in which orange or pink/red chromophores are formed from nicotine and its metabolites, using barbituric acid or 1,3-diethyl-2-thiobarbituric acid (DETB) as condensing reagents, respectively.¹² The colours that form may be read and compared with the colour produced by a known concentration of cotinine; alternatively, the colour produced may be extracted into ethyl acetate and assessed by eye in order to provide an immediate qualitative assessment of the smoking status of the individual. Using this method, Smith *et al.*¹³ have reported a sensitivity of 95% and specificity of 100%, compared with radioimmunoassay (RIA), for a qualitative assessment of smoking status.

The modified König reaction also has been used to develop a simple 'near patient' test, using DETB as a condensing reagent,^{14,15} for HPLC¹⁶ and in flow-injection¹⁷ analysis. In the original qualitative method of Peach *et al.*,¹² and subsequent HPLC procedures, the DETB-derived chromophores were extracted into ethyl acetate.

However, unlike the situation with cotinine, it was noted that some of the pink chromophores formed in smokers' urine samples were incompletely extracted into ethyl acetate. Therefore, it was argued that these probably were derived from much more polar metabolites of nicotine, and that the extractability of the reaction products might be considerably enhanced — and the sensitivity improved — if a more lipophilic barbituric acid derivative was employed. Hence the rationale for the synthesis of 1,3-dibutyl-2-thiobarbituric acid (DBTB).

Materials and methods

Potassium cyanide, chloramine-T and sodium acetate were purchased from Merck eurolab Ltd. (Lutterworth, UK). Acetic acid was purchased from Fisher Scientific Ltd. (Loughborough, UK). 1,3-dibutyl-2-thiobarbituric acid was synthesised using 1,3-dibutyl-2-thiourea and diethylmalonate, which were purchased from Aldrich (Gillingham, UK). Methanol, ethyl acetate and acetone were all of HPLC grade. The absorbencies of the urine samples derivatised with either DETB or DBTB were measured using a Cecil 9500 Super Aquarius. The identity of the synthesised DBTB was confirmed by nuclear magnetic resonance (NMR), using a Bruker AC 250.

Subjects

During a previous study, urine samples were collected from 251 patients attending the diabetic clinic at Ealing Hospital.¹² According to the gold-standard

cotinine RIA method, this population was judged to comprise 130 non-smokers and 120 smokers, with one result regarded as borderline. The current study utilised aliquots of the 224 urine samples (106 smokers, 118 non-smokers) remaining from the Ealing Hospital investigation, whose volumes were sufficient for the colorimetric analyses described below.

Samples were subjected to both qualitative assessment of smoking status and quantitative analysis by comparison with a standard cotinine preparation. Quantitative measurements were made before and after extraction with ethyl acetate, although the measurements taken after extraction with ethyl acetate were carried out on only 95 of the samples from RIA-confirmed smokers and on 115 samples from RIA-confirmed non-smokers. All samples were collected in accordance with the ethical code used at Ealing Hospital. All samples were deep-frozen until analysis and all analyses were carried out 'blind', without reference to the results of the RIA.

Preparation of 1,3-dibutyl-2-thiobarbituric acid

DBTB was prepared according to the method of Hahn *et al.*¹⁸ Briefly, 6.5 g sodium (washed with petroleum spirits and dried) was placed in a round-bottomed flask containing 75 mL methanol. 10 g N,N-dibutylthiourea (0.076 mol) and 17.2 g diethylmalonate (0.1 mol) were added to the flask, and the resulting reaction mixture was allowed to reflux for 36 h.

After this period, 50 mL distilled water was added and partly evaporated under pressure to remove any remaining methanol. The small amount of white precipitate (unreacted dibutylthiourea) formed during this evaporation was removed by filtration. The solution was diluted further with 100 mL distilled water, chilled on ice and then acidified with concentrated hydrochloric acid to between pH 1 and 2.

The resulting precipitate was collected and dried under reduced pressure to produce a light yellow powder of DBTB. The melting point range was between 56°C and 60°C. An NMR scan was carried out to confirm the identity of the compound — ¹H NMR (250 MHz; CDCl₃) 8 ppm 0.94 (6H, t, J 7.5, CH₃ x2); 1.35 (4H, m, CH₂CH₂CH₃); 1.6 (4H, m, CH₂CH₂CH₃) 3.7 (2H, s, O=CCH₂C=O); 4.3 (4H, t, J 7.5-NCH₂CH₂-).

Cotinine radioimmunoassay

For the purpose of this study, RIA was used as the gold-standard test. This is a well-documented method that can reliably distinguish between two categories; for example, an individual that is exposed to high levels of nicotine (i.e. a smoker) and an individual who is exposed to low levels of nicotine (i.e. a non-smoker).

Urinary cotinine concentrations were determined by RIA,¹¹ as described previously.¹² Aliquots (10 μ L) of the samples or cotinine calibrators (0.14–4.26 μ mol/L) were incubated overnight with cotinine antibody and radioactive iodine (¹²⁵I)-labelled cotinine. Bound and free fractions were separated using a polyethylene-glycol-assisted second antibody technique. The radioactivity in the bound fraction was counted, and cotinine concentrations were estimated using a four-parameter logistic curve-fitting programme. Within- and between-batch imprecisions were 5.1% and 7%, respectively.

König reaction

The total nicotine metabolite concentration was determined both qualitatively and quantitatively. Briefly, this involved adding 1 mL of individual nicotine metabolite, urine, or standard cotinine solution to 400 μ L sodium acetate buffer (pH 4.7). This was followed by addition of 200 μ L potassium cyanide (10% w/v) and 200 μ L chloramine-T (10% w/v). The solution was mixed well before the addition of condensing reagent (1 mL; DETB [1% w/v, water:acetone 50:50] or DBTB [1% w/v in acetone]). The reaction mixture was again mixed well and allowed to stand for 20 min. Absorbance measurements were made both before and after extraction with ethyl acetate (1 mL).

Qualitative assessment was by simple observation of a pink colour in the ethyl acetate layer. Absorbance of unextracted and ethyl-acetate-extracted samples was measured at 525 nm and 533 nm, respectively. A stock solution of 28 μ mol/L cotinine (5 μ g/mL) was used to determine the equivalent nicotine metabolite concentration. In addition, a sample blank was used, which comprised a mixture of urine samples from both self-reported smokers and non-smokers, and was assayed following the basic method, except that the chloramine-T was omitted.

Results

Urinary concentrations determined by cotinine RIA ranged from 0.06 μ mol/L to 0.97 μ mol/L (median:

0.1 μ mol/L) for non-smokers, and from 1.48 μ mol/L to 76.5 μ mol/L (median: 19.3 μ mol/L) for smokers.

Urinary concentrations determined using DETB ranged from <0.1 μ mol/L to 12.9 μ mol/L (median: 1.25 μ mol/L) for non-smokers, and 4.32 to 282 μ mol/L (median: 45 μ mol/L) for smokers.

Urinary concentrations determined using DBTB without extraction ranged from <0.1 μ mol/L to 9.8 μ mol/L (median: 4.29 μ mol/L) for non-smokers, and 5.17 μ mol/L to 324 μ mol/L (median: 30.3 μ mol/L) for smokers.

When measurements were made post-extraction (DBTB only), results ranged from <0.1 μ mol/L to 12.6 μ mol/L (median: 0.91 μ mol/L) for non-smokers, and 2.56 μ mol/L to 212 μ mol/L (median: 23.8 μ mol/L) for smokers (Table 1).

The optimum cut-off point between smokers and non-smokers using DBTB was determined by receiver operator characteristic (ROC) curve analysis¹³ from a consideration of sensitivity and specificity when compared with the gold-standard RIA determination. Sensitivity was calculated as the proportion of true positive (TP) test results obtained in subjects who smoked (i.e. TP/TP + FN [false negatives]), whereas specificity was calculated as the proportion of true negative (TN) results obtained in subjects who did not smoke (i.e. TN/TN + FP [false positives]). Overall test efficiency was calculated as TP + TN/TP + FP + TN + FN. The cut-off point for DBTB unextracted was determined as 8.5 μ mol/L, compared with 4 μ mol/L for samples extracted in ethyl acetate.

Using DETB, qualitative assessment of smoking status gave a sensitivity of 94% and specificity of 100%. Using DBTB, sensitivity and specificity were 100% and 99%, respectively. There was an overall improvement in efficiency from 97% with DETB to 99.5% with DBTB. When the samples were analysed quantitatively, results for DETB-unextracted samples gave a sensitivity and specificity of 98%. DBTB-unextracted samples produced a sensitivity and specificity of 95% compared with results obtained from samples using DBTB extracted in ethyl acetate, which gave a sensitivity and specificity of 96% (Table 2).

Table 1. Urinary cotinine and TNM values in smokers and non-smokers (μ mol/L)

	Non-smokers		Smokers	
	Median	Range	Median	Range
RIA	0.1	0.06–0.97	19.3	1.48–76.5
DETB	1.25	<0.1–12.9	45	4.3–282
DBTB	4.29	<0.1–9.8	30.3	5.2–324
DBTB (post-extraction)	0.91	<0.1–12.6	23.8	2.6–212

Table 2. Sensitivity and specificity of barbiturate derivatives, compared with the RIA gold standard

	Sensitivity	Specificity
Qualitative		
DETB	94%	100%
DBTB	100%	99%
Quantitative		
DETB	98%	98%
DBTB (post-extraction)	95%	95%
DBTB	96%	96%

Amongst the smokers, correlation between urinary cotinine measurement (RIA) and the colorimetric procedures was determined using Spearman's Rank analysis. DBTB extracted ($n = 95$): $r_s = 0.84$, $P < 0.01$ (Figure 1); DBTB unextracted ($n = 106$): $r_s = 0.790$, $P < 0.01$. Correlation between DETB and DBTB unextracted ($n = 106$) gave $r_s = 0.871$, $P < 0.01$.

Discussion

There is a need for a quick, simple, inexpensive test to assess smoking status in a variety of settings. Several workers have attempted to employ the König reaction for this purpose. The approach employed here — that is, attempting to improve assay sensitivity by use of a more non-polar derivatisation reagent, allied to extraction of the coloured product — yielded good

results. Qualitative assessment of smoking status was clearly superior to quantitative determination. The increase in partitioning between the aqueous and ethyl acetate phases, obtained using DBTB, improves distinction between non-smoker and smoker, from an overall efficiency of 97% with DETB to 99.5% with DBTB. However, this distinction is notoriously difficult to make because no individuals truly can be said to not be exposed to tobacco smoke; and in any study of this type, the numbers of subjects appearing at the cut-off point is usually small.

Use of this compound offers clear advantages, both for HPLC analysis and for use in small near-patient devices for monitoring exposure to environmental tobacco smoke.¹⁹ Correlation between RIA and colorimetric methods is good, but the results clearly are skewed by one or two subjects who had levels of total nicotine metabolites proportionately higher compared with cotinine. The implication is that these individuals may metabolise nicotine differently to the majority.

This is in accordance with the recent finding of null alleles for the cytochrome P₄₅₀ enzyme CYP2A6, which is important in the metabolism of nicotine.²⁰ Individuals who cannot metabolise nicotine, or who metabolise nicotine poorly via the P₄₅₀ pathway, may have greater amounts of other nicotine metabolites, which are measured in the König reaction but not by RIA.

Recent work suggests that nicotine is metabolised differently in the pregnant and non-pregnant

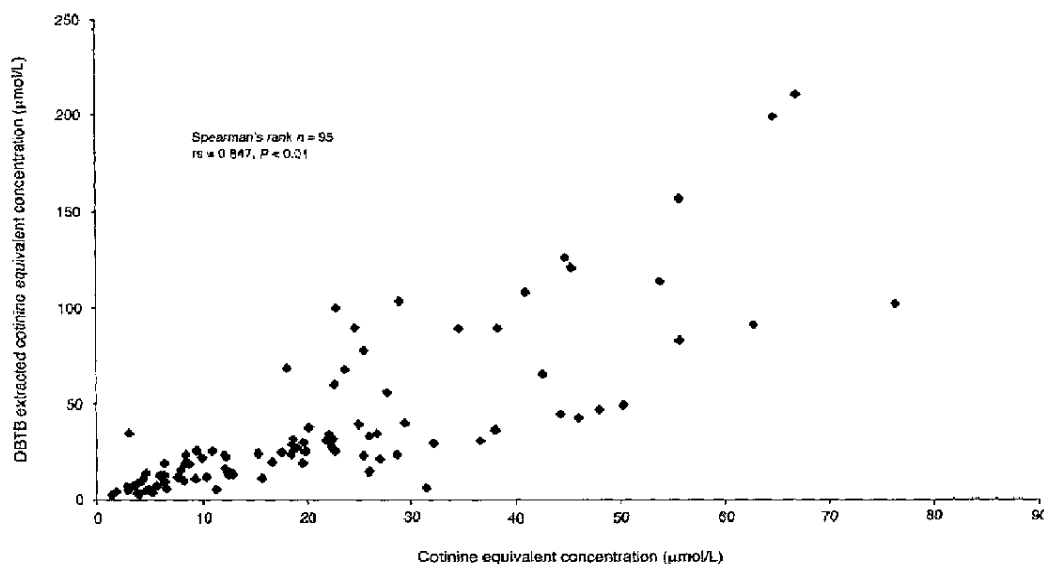


Fig. 1. Correlation between DBTB (extracted) and cotinine RIA.

individual,²¹ and that there are inter-racial differences in nicotine metabolism.²² The novel derivative described in this paper provides an improved, simple, inexpensive and near-perfect test for the determination of smoking status.

This project was supported by a research grant from the Institute of Biomedical Science.

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